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Removal of organic content from diesel exhaust particles alters cellular responses of primary human bronchial epithelial cells cultured at an air-liquid interface

Annalicia Vaughan¹, Svetlana Stevanovic², Leanne E Morrison¹, Ali Mohammad Pourkhesalian², Mostafizur Rahman², Ali Zare², Branka Miljevic², Felicia Goh¹, Vandana Relan¹, Rayleen V. Bowman¹, Kwun M. Fong¹, Steven E. Bottle², Zoran D. Ristovski², Ian A. Yang¹

¹ *The University of Queensland Thoracic Research Centre, The Prince Charles Hospital, Brisbane, Australia*

² *International Laboratory for Air Quality and Health, The Queensland University of Technology, Brisbane, Australia*

Address correspondence to: Annalicia Vaughan, UQ Thoracic Research Centre, The Prince Charles Hospital, Rode Rd, Chermside, 4032, Queensland, Australia. E-mail: annalicia.vaughan@uqconnect.edu.au

Abstract

Background: Exposure to air pollutants, including diesel particulate matter, has been linked to adverse respiratory health effects. Inhaled diesel particulate matter contains adsorbed organic compounds. It is not clear whether the adsorbed organics or the residual components are more deleterious to airway cells. Using a physiologically relevant model, we investigated the role of diesel organic content on mediating cellular responses of primary human bronchial epithelial cells (HBECs) cultured at an air-liquid interface (ALI).

Methods: Primary HBECs were cultured and differentiated at ALI for at least 28 days. To determine which component is most harmful, we compared primary HBEC responses elicited by residual (with organics removed) diesel emissions (DE) to those elicited by neat (unmodified) DE for 30 and 60 minutes at ALI, with cigarette smoke condensate (CSC) as the positive control, and filtered air as negative control. Cell viability (WST-1 cell proliferation assay), inflammation (TNF- α , IL-6 and IL-8 ELISA) and changes in gene expression (qRT-PCR for HO-1, CYP1A1, TNF- α and IL-8 mRNA) were measured.

Results: Immunofluorescence and cytological staining confirmed the mucociliary phenotype of primary HBECs differentiated at ALI. Neat DE caused a comparable reduction in cell viability at 30 or 60 min exposures, whereas residual DE caused a greater reduction at 60 min. When corrected for cell viability, cytokine protein secretion for TNF- α , IL-6 and IL-8 were maximal with residual DE at 60 min. mRNA expression for HO-1, CYP1A1, TNF- α and IL-8 was not significantly different between exposures.

Conclusion: This study provides new insights into epithelial cell responses to diesel emissions using a physiologically relevant aerosol exposure model. Both the organic content and residual

components of diesel emissions play an important role in determining bronchial epithelial cell response *in vitro*. Future studies should be directed at testing potentially useful interventions against the adverse health effects of air pollution exposure.

Key words: diesel emissions, diesel organic content, bronchial epithelial cells, air-liquid interface, inflammation

Background

Epidemiological studies have linked air pollution exposure to increased respiratory and cardiovascular morbidity and mortality [1-3]. The World Health Organization has reported that 7 million deaths world-wide in 2012 were attributed to air pollution exposure, with 31% of these deaths from respiratory disease [4]. The three main air pollutants are particulate matter, ozone and nitrogen dioxide [3]. Research has shown a strong association between particulate matter exposure and adverse respiratory health effects [5-7]. Diesel emissions are one of the major contributors to particulate matter [3]. Diesel particulate matter (DPM) consists of partially combusted carbon particles, which have organic content absorbed to the particle surface [3]. DPM can vary in composition and size depending on the emissions source [3]. Inhaled diesel particles of smaller size display higher deposition in the lower airways, higher percentage of organic content and increased surface area to mass ratio, making smaller particles more biologically active and toxic within the lungs [8].

The first line of defence against inhaled DPM is the bronchial epithelium, a cellular structural barrier lining the airways [9]. The main mechanisms of defence mediated by the bronchial epithelium are mucociliary clearance, initiation of inflammatory response and degradation of inhaled agents [9]. When DPM are inhaled into the airways, the ultrafine DPM can penetrate the mucus layer, then internalise and aggregate in the epithelium [10, 11]. *In vitro* studies have shown

this DPM aggregation triggers cellular responses including the secretion of pro-inflammatory cytokines TNF- α , IL-8 and IL-6 [10-15], as well as triggering xenobiotic metabolism [16, 17], oxidative stress [18] and ultimately loss of cell viability [19, 20]. Xenobiotic metabolism is the degradation of foreign agents within an epithelial cell by a xenobiotic metabolising enzyme, cytochrome P450 1A1 (CYP1A1). CYP1A1 expression is triggered by the activation of the aryl hydrocarbon receptor (AhR) by diesel particulate organic content. These defence mechanisms of bronchial epithelial cells are critical for normal functioning of the lungs; however, very little is known about how exposure to DPM alters these defensive cellular responses.

A major challenge in aerosol toxicity research is finding an accurate *in vitro* representation of diesel exposure to the airways *in vivo*. Previous studies have used a conventional, submerged cell exposure model, in which DPM are dissolved in cell culture media and administered to a monolayer of immortalised epithelial cells [21]. However, this model lacks the physiological relevance needed for diesel toxicity studies, as the diesel composition is potentially altered by the suspension in media [20] and the interaction between the diesel and the cell layer is not occurring directly at an air-liquid interface (ALI). Additionally, the use of immortalised bronchial epithelial cells is a limitation, as these cells may not accurately represent the mucociliary phenotype of the *in vivo* cell population [9]. The use of primary human bronchial epithelial cells (HBECs) cultured at ALI, and differentiated into a mucociliary epithelial cell layer, effectively mimics the *in vivo* environment of the bronchial epithelium [22-24]. This more physiologically relevant platform models the inhalation of pollutants *in vivo*, and enables the composition of diesel emissions to be modified experimentally, in order to accurately assess the effects of different DPM components on bronchial epithelial cell responses.

The aim of this study was to investigate the effect of removing organic content from diesel emissions, on the cellular responses of primary HBECs cultured at (ALI). It was hypothesised that

exposure to residual diesel emissions (without organic content) would result in attenuated HBECs responses, when compared to neat (unmodified) diesel emission exposure.

Methods

Cell culture

Primary HBECs were isolated and cultured from surgical resection tissue (left lower lobe) donated, with written informed consent, by a 57 year old male patient with lung adenocarcinoma who was a current smoker with a 70 pack-year history of smoking and forced expiratory volume (FEV₁) of 83% predicted. This study was approved by the Human Research Ethics Committees of The Prince Charles Hospital and The University of Queensland. Briefly, a bronchial ring was isolated from the lung resection specimen and incubated in a dissociation mix containing Pronase (Roche, Penzberg, Germany), Minimal Essentials Media-alpha (MEM α , Invitrogen, USA), amphotericin B (Invitrogen), penicillin, streptomycin and L-glutamine (PSG, Invitrogen) for 24 hours at 4°C, based on a published method [25]. Briefly, the epithelial cell layer was then harvested from the bronchial ring, and centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in 4 mL of Keratinocyte Serum-Free Media (KSFM, Invitrogen), expanded in submerged culture until confluent and stored in liquid nitrogen until needed.

Primary HBECs were grown and differentiated using a Bronchial-Air Liquid Interface (B-ALI) BulletKit (Lonza, Basel, Switzerland), according to the manufacturer's instructions. Briefly, the HBECs were seeded onto a porous collagen-coated membrane on a 6.5mm Corning Transwell® (Corning®, Corning, USA) insert at a density of 50,000 cells per insert. The cells were grown until confluent in B-ALI growth media on the apical and basal surfaces. The apical media was then removed to achieve air lift, and the basal media was replaced with supplemented B-ALI differentiation media. The cells were maintained at ALI for at least 28 days before exposure to

diesel exhaust. The basal media was changed 3 times per week, and the apical surface of the cell layer was washed at least once a week with 150 μ L of phosphate buffered saline (PBS) solution.

HBEC characterization

Mucus secretion

Alcian blue staining was used to qualitatively assess the presence of mucus-secreting HBECs. Prior to staining, the apical surface of the cell layer was washed with 150 μ L of PBS. 100 μ L of Alcian blue dye was pipetted onto the cell layer and incubated for 30 seconds at room temperature. The dye was removed and the apical surface was photographed at 10x magnification.

Immunofluorescence staining for β -tubulin

The presence of ciliated HBECs was confirmed using immunofluorescence staining targeting β -tubulin. The Transwell® membrane was fixed with cold methanol and incubated with an anti- β -tubulin antibody conjugated to fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI). The membrane was incubated with each stain separately for 5 minutes at 37°C and washed in between stains with 150 μ L of PBS. The Transwell® membrane was then cut away from the insert and mounted onto a glass slide. The apical surface of the cell layer was observed under fluorescent microscopy (Axio Imager Z1, Zeiss, Jena, Germany) and photographed using AxioVision LE microscopy software (Zeiss).

Diesel emissions generation and characterization

Diesel emissions were generated on a Euro III common rail Cummins diesel engine, coupled to a dynamometer. Fuel used in this study was commercial diesel purchased from Caltex. Running mode was at 25% load at 1500 rpm. The specifications of the engine and dynamometer are shown in Table 1, and the diesel emission set-up is shown in Figure 1.

The raw exhaust was subjected to one stage dilution through partial flow dilution. A centrifugal pump drew HEPA-filtered ambient air into the dilution tunnel. Gas analysers, which consisted of a NDIR (Non-destructive infra-red) CAI 600 series CO₂ and CO analyser and a CAI 600 series CLD (Chemiluminescence detector) NO_x analyser, measured CO, CO₂ and NO_x concentrations before the dilution system. To determine the dilution ratio, a SABLE CA-10 CO₂ analyser was used to measure CO₂ concentrations after the dilution. A Scanning Mobility Particle Sizer (SMPS; TSI 3080 Electrostatic classifier, with a 3025 Condensation Particle counter measured the size distribution and number concentration of DPM. The semi-volatile organic fraction was removed by passing the polydisperse aerosol stream through a TSI 3065 thermodenuder. To take into account particle losses inside the thermodenuder [26], all the tests were done with the thermodenuder in the system. Neat (nDE) and gas-phase (gDE) diesel emissions were sampled through the thermodenuder at room temperature, while residual diesel emissions (rDE) were generated by sampling through the thermodenuder set to 300°C [27]. Dustrak (model TSI 8520) was used for PM_{2.5} measurements (particulate matter measuring $\leq 2.5\mu\text{m}$ in diameter). Dustrak mass concentration data were converted to gravimetric mass concentrations as measured by the tapered element oscillating microbalance based on the equation provided in [17].

Central flow of 1 L/min was used to avoid agglomeration and loss of small particles. The tubing was kept as short as possible to further minimize diffusion losses. Three processes influence the deposition efficiency of the CULTEX: sedimentation, diffusion and electrical forces. Deposition of nanoparticles (diameter $< 0.1\mu\text{m}$), is mainly controlled by electrical forces. The deposition efficiency calculated was 0.81. Detailed discussion on the theoretical and practical aspect of the efficiency of the CULTEX® RFS system is provided in [23].

Cell exposure

Primary HBECs were cultured at ALI and exposed to diesel emissions through the CULTEX[®] Radial Flow System (CULTEX[®] Laboratories GmbH, Hannover, Germany), which contains three cell exposure chambers allowing simultaneous exposure in triplicate. The apical module directs air flow at 0.025 L/min directly over the cell layer using an external vacuum pump. The Transwell[®] insert containing the cell layer sits on the basal module, which is filled with 25mL of PBS solution heated to 37°C by an external water bath. HBECs were exposed in triplicate with neat diesel emissions (nDE) or residual diesel emissions (rDE) with organics removed for 30 and 60 minutes. The negative control samples were HBECs exposed to filtered laboratory air (FA) for 30 minutes and 60 minutes. The positive control samples were exposed to cigarette smoke condensate (CSC) diluted in apical media at 4mg/μL and incubated for 3 hours at 37°C. The CSC was purchased from Murty Pharmaceuticals Inc. (Lexington, KY). Baseline readings of cell responses were measured from unchallenged incubated HBECs that underwent none of the experimental handling.

Cytokine secretion

Enzyme-linked immunosorbent assays (ELISA) were used to measure the level of IL-8, TNF-α and IL-6 secretion from primary HBECs in response to diesel emission exposure at ALI. After completion of exposure, 250μL of B-ALI differentiation media was added to the apical surface of the cell layer and incubated at 37°C in 5% CO₂ for 24 hours. The supernatants were then removed from the cell layer and stored at -80°C until ELISA analysis. The concentrations (pg/mL) of IL-8, TNF-α and IL-6 were determined using Quantikine ELISA assay kits (R&D systems, Minneapolis MN, USA), according to the manufacturer's instructions. Colour intensity was measured and quantified using a microplate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany) with software for quantification (Omega v1.2). The standard curves were generated using a 4-parameter logarithmic curve fit in Prism6.0c. For accurate comparison between experimental

groups, the cytokine concentrations were corrected for cell viability to show data representative of a 100% viable cell layer.

Cell viability

After removal of the supernatant, a water-soluble tetrazolium-1 (WST-1) cell proliferation assay (Roche Applied Sciences, Penzberg, Germany) was performed as a measure of cell viability. WST-1 is a salt solution that is metabolised by mitochondrial dehydrogenase to produce formazan, causing a measurable colour change. Reagent stock was diluted 1:10 with B-ALI differentiation media, added to the surface of the cell layer in the Transwell[®], and incubated at 37°C in 5% CO₂. After 3 hours, WST-1 sample solution was transferred to 96-well plate alongside incubated control wells containing 10µL of WST-1 plus 90µL of B-ALI differentiation media. Absorbance was measured and quantified using a microplate reader (FLUOstar Omega) with software (Omega v1.2), at wavelength 450nm against background control (blank) and corrected for interference at 620nm. Cell viability was expressed as percentage relative to the baseline samples, which were deemed to be 100% viable.

Gene expression

RNA extraction

Total RNA was extracted from cells using the Qiagen AllPrep kit (Qiagen, Limburg, Netherlands). The cell layer was homogenised by adding 200µL of RLT buffer to the apical surface of the cell layer, placed on a shaker and incubated at room temperature for 20 minutes. The homogenised lysate samples were collected into Eppendorf tubes and an additional 150µL of RLT buffer was added to make up the 350µL needed for RNA extraction. The samples were stored at -80°C until RNA extraction. During the RNA extraction protocol, the RNA samples were cleaned of genomic DNA using an RNase-free DNase kit (Qiagen) as per the manufacturer's instructions. RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, DE,

USA). Quality assessment was performed by measurement of A260/A280 (>1.75 indicating good quality) and A260/A230 ratios (>1.75 indicating good quality), representing RNA/protein and RNA/contaminant absorbance, respectively.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to assess the changes in HO-1, CYP1A1, IL-8 and TNF- α gene expression after diesel exposure. cDNA was prepared by reverse transcribing extracted RNA samples using Superscript III First Strand Synthesis kits (Qiagen) as per the manufacturer's instructions. TaqMan Gene expression assays targeting HO-1 (Hs01110250_m1), CYP1A1 (Hs01054797_g1), TNF α (Hs01113624_g1) and IL-8 (Hs00174103_m1) were performed, and compared to the expression of a housekeeper gene, GAPDH (Hs02758991_g1). RT-PCR was performed using the ViiA 7 Real-time PCR system (Applied Biosciences, Foster City, CA, USA) with the following PCR cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The change in gene expression was expressed as fold change relative to baseline levels using the comparative quantification algorithm, $-\Delta\Delta C_t$.

Statistical analysis

A one-way ANOVA (two tailed) with Tukey's multiple comparisons post-test between all experimental groups was used to assess the effects of the experimental challenge on the measured cellular responses (cytokine secretion, cell viability or gene expression). In the graphs, an asterisk (*) directly above the bar indicates a statistically significant difference when compared to the filtered air control. A connecting line between two bars and an asterisk above indicates a significant difference between the highlighted aerosol exposures. As positive control, exposure to CSC was compared with the baseline control for significance difference. Statistical analysis was performed using Prism v6.0c. P values <0.05 (two-tailed) were considered statistically significant.

Results

HBEC differentiation

ALI cultures of primary human bronchial epithelial cells were used to generate a mucociliary epithelial cell layer that is representative of the *in vivo* situation. Figure 2 shows immunofluorescence stains of the cell layer after 0 and 28 days at ALI at 20x magnification. DAPI is a nucleic acid stain used to show the overall cell population by highlighting the nuclei. The cilia were stained using a β -tubulin antibody conjugated to the green fluorescent compound FITC. Comparison between day 0 and day 28 confirmed the presence of cilia after differentiation at ALI, shown by the positive β -tubulin stain at day 28 and not day 0. Figure 3 shows Alcian blue stains of the apical surface of the cell layer at 10x magnification. Alcian blue targets mucopolysaccharides and the increase in blue colour intensity between day 0 and day 28 at ALI confirmed the mucosecretory phenotype of the cell layer.

Diesel exhaust generation and characterization of particles

Size distribution and particle number concentrations ($\#/cm^3$) of emitted particles can be seen in Figure 4. Emitted mass of particles corrected for the losses inside the thermodenuder as well as concentration of NO, NO₂, CO and CO₂ in the raw gas are presented in the Table 1. The particle number concentration, mass concentration and gas concentration remained consistent between the 30 and 60 minute exposures for both nDE and rDE. There was a notable loss in the concentration of particles when comparing between the nDE and rDE exposures, which is due to thermophoretic losses. Mass concentration dropped as well, but the decrease in the mass was not as large, since the carriers of the mass are mainly larger particles whose number was reduced by only 10-20%.

Cell viability

The percentage of viable cells was assessed using a WST-1 cell proliferation assay for primary HBECs exposed to nDE and rDE for 30 and 60 minutes. A 30 minute exposure to nDE caused a significant reduction in HBEC viability when compared to the filtered air negative control ($P=0.01$), whereas exposure to rDE resulted in no change in viability (Figure 5). A 60 minute exposure to nDE resulted in a similar magnitude of reduction in cell viability as the 30 minute exposure; however this did not reach statistical significance ($P=0.08$). Exposure to the rDE for 60 minutes resulted in a statistically significant reduction in HBEC viability when compared to filtered air ($P=0.0001$) and 30 minutes rDE exposure ($P<0.0001$). As a positive control, high dose CSC exposure showed the largest decrease in cell viability when compared to baseline and filtered air controls ($P=0.0001$).

Cytokine secretion

Figure 6 shows the cytokine secretion levels for IL-8, TNF- α and IL-6 quantified using ELISA analysis of supernatant collected 24 hours after exposure and corrected for the percentage of viable cells. 30 minute exposure to nDE or rDE did not significantly alter IL-8, TNF- α and IL-6 secretion when compared to the filtered air control. Similarly, there was no significant change in IL-8, TNF- α and IL-6 secretion after a 60 minute exposure to nDE, compared to the filtered air control ($P>0.05$). In contrast, there were statistically significant increases in IL-8, TNF- α and IL-6 secretion after 60-minute exposure to rDE when compared to filtered air ($P=0.007$, $P=0.01$ and $P<0.0001$, respectively). In the 60 minute exposures, TNF- α and IL-6 concentrations were also significantly higher after exposure to rDE, compared to nDE ($P=0.008$ and $P=0.0002$, respectively). High dose CSC exposure resulted in statistically significant increases in IL-8 and TNF- α secretion, when compared to filtered air ($P<0.0001$). For accurate comparison between experimental groups, the cytokine concentrations were corrected for cell viability to show data representative of a 100% viable cell layer. Uncorrected data is shown in supplementary figure 1.

Gene expression

Gene expression analysis was performed using quantitative real-time PCR targeting CYP1A1, HO-1, TNF α and IL-8 mRNA. The data were normalised to the housekeeper gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expression levels were calculated relative to the baseline control group. Figure 7a shows the relative gene expression levels of CYP1A1, which is a xenobiotic metabolizing enzyme responsible for metabolizing polycyclic aromatic hydrocarbons (PAHs). Whilst exposure to rDE for 30 or 60 minutes tended to increase CYP1A1 gene expression, there were no significant changes in gene expression after 30 or 60 minute exposure to nDE or rDE ($P>0.05$). For the positive control, high dose CSC exposure resulted in a marked increase in CYP1A1 expression ($P<0.0001$).

For gene expression of HO-1 (Figure 7b), an antioxidant protein expressed in response to oxidative stress, 30 minute exposure to nDE or rDE did not significantly change HO-1 expression ($P>0.99$ and $P=0.99$, respectively). In the 60 minute exposures, HO-1 expression increased with nDE and to a lesser extent rDE; however these did not reach statistical significance when compared to filtered air ($P=0.3$ and $P=0.95$, respectively). Furthermore, high dose CSC did not alter HO-1 expression in these experiments ($P>0.99$).

The gene expression of inflammatory cytokines TNF- α and IL-8 were measured in conjunction with levels of protein secretion. 30 minute exposure to nDE did not change significantly in TNF- α and IL-8 mRNA expression, compared to the filtered air control ($P=0.87$ and $P=0.91$, respectively). Exposure to rDE for 30 minutes did not alter TNF- α or IL-8 expression ($P>0.99$ and $P=0.99$, respectively). 60 minute exposure to nDE and rDE did not change in the relative expression of TNF- α and IL-8 ($P>0.05$). High dose CSC caused minor changes in TNF- α and IL-8

mRNA expression, but both were not statistically significant when compared to the filtered air controls ($P=0.54$ and $P=0.94$, respectively).

Discussion

Main findings

Diesel emissions are a major source of air pollution within the urban environment and long-term exposure has been linked to an increase in respiratory disease morbidity and mortality [1-3]. Diesel emissions are made up of gas and particulate matter, the latter of which is considered a highly toxic component [3]. The diesel particulate composition can vary greatly depending on the emissions source [5-7], therefore it is important to understand the potential those different components have in triggering biological responses. The primary objective of this study was to investigate the effect removing the diesel organic compounds from DPM has on primary HBECs using direct air-to-cell exposure at ALI. This study has demonstrated that the removal of organic content from diesel emissions can alter cellular responses with three main findings. Firstly, nDE and rDE triggered differential effects on the HBEC responses (cell viability, inflammation, xenobiotic metabolism and oxidative stress) when comparing between 30 and 60 minute exposures; cell viability changes were comparable to cytokine secretion, but not the levels of HO-1 expression (oxidative stress marker); and finally, in contrast to previous literature [16, 28], there was an increase in CYP1A1 expression in the absence of organic content. These findings have built upon the current understanding of how bronchial epithelial cells respond to diesel emissions *in vitro* and offer new insight into how diesel emissions may trigger adverse health effects in relation to epithelial cell response.

The effect of removing organic content on diesel emissions toxicity as measured by cell viability

Previous studies have shown that exposure to diesel emissions can be highly toxic to airway epithelial cells both *in vitro* [20] and that the organic content present on the diesel particulate matter plays a key role in this toxicity [19]. In accordance with previous studies we showed reduced cell viability after exposure to nDE [20]. The removal of organic content appeared to attenuate the effect on cell viability for the 30 minute exposure. In contrast, the removal of organic content resulted in a stronger reduction in cell viability after the 60 minute exposure, compared to the 30 minute exposure and the nDE exposure. This somewhat unexpected result challenges the notion that organic content is the major contributor to the toxicity of diesel emissions. Previous research has shown that cell viability can be attenuated with the removal of organic content [11, 16, 19, 28], however many of these studies used the submerged culture method for diesel emission exposure in which the diesel components are suspended in media, with the possibility that suspension of diesel allows for alteration in chemical composition [21]. Because we used direct aerosol exposure to measure the effects of organic content on diesel toxicity, we have minimised interference by apical cell culture media. These methods provide evidence for the contribution of other diesel components (residual after the removal of organic content) to the toxic effects of diesel emissions exposure on human bronchial epithelial cells.

The effect of removing diesel organic content on oxidative stress and inflammation

Diesel emissions toxicity is associated with oxidative stress, a common adverse effect of environmental toxins on cells [29]. Oxidative stress can be caused by inflammatory activity [30], xenobiotic metabolism [31] and/or endogenous reactive oxygen species (ROS) from DPM [32], and this oxidative stress often leads to a reduction in cell viability [33-35]. HO-1 is a cytoprotective antioxidant protein and increases in expression in response to oxidative stress [36, 37]. After the 30 minute exposure to nDE and rDE, there were limited HBEC responses of

cytokine (protein or mRNA) secretion, and no major changes in HO-1 mRNA expression. After the longer 60 minute exposures, cytokine protein secretion increased significantly with rDE, whereas cytokine mRNA expression and HO-1 mRNA expression did not change with either rDE or nDE. These results highlight the importance of diesel emissions composition and the differential effects of organic content on HBEC responses after 30 and 60 minutes exposure.

The effect of removing diesel organic content on xenobiotic metabolism

Previous research has shown that the presence of organic content on diesel emission particles trigger the expression of xenobiotic metabolizing enzyme, CYP1A1 [16, 28]. The classical method of CYP1A1 expression activation, in reference to diesel emission exposure, requires the activation of the AhR receptor by diesel organic content [31]. We showed that nDE exposure had limited effect on CYP1A1 expression. This could be due to inadequate exposure duration, as in previous studies CYP1A1 expression increased after 60 minutes of diesel emission exposure [18]. However, there was some increase in CYP1A1 expression after exposure to rDE (without organics) when compared to both the filtered air controls and the nDE, although this did not reach statistical significance. In accordance with this, a study by Totlandsdal and colleagues (2012) used the conventional particle/media suspension method of exposure to show that residual diesel particles (without organics) can trigger an upregulation of CYP1A1 expression from the immortalized bronchial epithelial cell line BEAS-2B [19]. This suggests that during diesel emissions exposure, the upregulation of CYP1A1 expression is not dependent only on the organic content and may be triggered by other DPM components. Although direct conclusions regarding the mechanistic background of toxic behaviour of residual diesel particles cannot be drawn from these measurements, the results imply that it is likely that some new pathways may be responsible for the observed effects.

The advantages of direct air-to-cell diesel emissions exposure

To our knowledge, this study is the first to test the effects of different diesel emissions components on differentiated HBECs using a direct air-to-cell exposure method. Much of the previous research in this area has used an experimental model where DPM are suspended in media and administered to a submerged immortalized epithelial cell monolayer [17, 28]. This experimental model lacks true physiological relevance [21] as the physical and chemical composition of the DPM change in suspension [18, 21] and the bronchial epithelial cell monolayer is not representative of the airway epithelium *in vivo*. In contrast, we observed successful differentiation of primary HBECs into a mucociliary epithelial cell layer. This culture technique then provided the platform for direct diesel emission exposure through the CULTEX® RFS and supported closer modelling of the adverse effects of diesel emissions on airway epithelial cells. Through use of physiologically relevant exposure model, our experiments provide new insight into the mechanisms by which air pollution can affect HBECs.

Organic content on PM has been shown to confer toxicity as a result of its chemical composition and related reactivity [32, 38]. Hence, our results associated with nDE exposure are not surprising. However, at the same time, the organic coating is disabling the reactivity of the carbon surface. By removing this coating, the large carbon surface area and its many active sites become exposed, and may trigger other reactions within cells with a longer duration of exposure. Another potential consideration is that thermodenuded particles may alter the stability of soot agglomerates, leading to further decomposition by the breakage of solid bridges of other substructures formed during the dilution process and related Brownian coagulation [39]. The probability of fragmentation of agglomerated diesel particles under various conditions depends on many factors and should be further explored for its potential role in electrochemical processes in cell fluid suspensions. Furthermore, in either of these cases, the dose or a threshold value may be a key determinant in switching on a certain cellular response, which may be relevant for different responses of residual diesel after 30 minutes and 60 minute exposures. In addition, the kinetics of reactions involving

organic compounds and activated soot surface area, as well as smaller units of diesel agglomerates, may be completely different, having important implications for cells being ultimately coupled to the exposure time.

Limitations

The study of aerosol toxicity in the airways has always been challenging due to the complex composition of different pollutants and an inaccurate representation of the airway epithelium. Although this study has used direct aerosol exposure to a differentiated epithelial cell layer to attempt to overcome these major limitations, some limitations remain. Firstly, the primary HBECs used were established from airway tissue collected from a single patient with lung cancer who smoked. Results obtained in this experimental system may not be generalisable to other individuals. While these considerations do not interfere with the internal validity of the findings, additional experiments on other primary cell lines should be performed in the future. Secondly, some consideration needs to be made for the time-point for gene expression analysis. The HBECs were lysed and RNA extracted after the 24 hour incubation and supernatant collection. Each gene of interest may have different expression patterns in response to diesel emissions exposure. For CYP1A1 mRNA expression analysis, the time-point of gene expression analysis was validated by the CSC exposure (positive control), which showed a significant increase in mRNA expression. This result showed that regulated changes in CYP1A1 mRNA expression are sustained 24 hours after diesel emission exposure. However, this significant difference was not demonstrated for HO-1, TNF α and IL-6 mRNA expression. Lastly, it has been shown that apoptotic processes can suppress inflammation [40], therefore we considered it important to correct cytokine secretion for loss of cell viability in order to provide a more biologically relevant snapshot of responses under various diesel exposure conditions per viable cell.

Conclusions

The results of this study indicate that both the organic content and residual components of diesel emissions play an important role in mediating bronchial epithelial cell response *in vitro*. Cell viability and pro-inflammatory cytokine secretion (IL-8 and TNF- α) showed comparable changes, indicating that inflammatory responses may be a key mechanism of response to diesel emissions, more so than oxidative stress. While rDE (without organic content) has the ability to trigger CYP1A1 expression, this may also be regulated by the residual components of DPM. Together these findings provide new insight into bronchial epithelial cell responses to diesel emissions. Future studies could be directed at defining the active signalling pathways and testing interventions against the adverse health effects of air pollution.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

AV cultured the HBECs, participated in the diesel emission exposures, conducted the cell response tests and drafted the manuscript. LM assisted with developing the cell culture methods and performing cell response testing. SS participated in the diesel emissions exposure, the design and coordination of the study, and helped to draft the manuscript. AMP, MR and AZ participated in diesel emissions generation and exposure testing. BM, ZR, SB, KF, RB and IY contributed to concept and experimental design of the project, as well as critical analysis of the data. FG and VR assisted with gene expression assays and advised on data analysis. All authors read and approved the final manuscript.

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Figure 1: Set-up for diesel emissions generation and analysis. Exhaust gas is first sampled from the exhaust line, and exhaust gas is then mixed with HEPA (High-Efficiency Particulate Air Filter) air. After dilution stage, it passes through a thermo-denuder where the volatile organic fraction of DPM is stripped off. A Scanning Mobility Particle Sizer (SMPS) gives the size distribution of the exhaust gas. Dustrak measures particulate mass. The CULTEX system is used for cell exposure. CO₂ analysers are used before and after dilution system to calculate the dilution ratio.

Figure 2: Immunofluorescence staining of primary HBECs for cilia using an anti- β -tubulin antibody conjugated with FITC, co-stained with DAPI on HBECs at day 0 (a-c) and day 28 (d-f). From top to bottom: DAPI, β -tubulin and merged, respectively.

Figure 3: Alcian blue stain for mucus secretion from primary HBECs at day 0 and day 28. a-b) Apical surface of the cell layer prior to staining; c-d) Apical surface of the HBEC layer after Alcian blue stain.

Figure 4: Size distribution of neat diesel emissions (nDE) and residual diesel emissions (rDE). Particle number distributions were measured with a scanning mobility particle sizer (SMPS) consisting of a TSI 3071A classifier (EC) and a TSI 3010 condensation particle counter (CPC). Particles within a 10-400nm size range were measured. For the neat diesel tests, 15 SMPS scans were taken, and at least 15 scans were taken for tests involving residual diesel exhaust.

Figure 5: WST-1 assay measuring the cell viability of primary HBECs after exposure to filtered air (FA), neat diesel emissions (nDE) and residual diesel emissions (rDE) for 30 and 60 minutes, and cigarette smoke condensate (CSC) for 3 hours. * indicates a statistically significant difference ($P < 0.05$). Error bars indicate SEM ($n=3$). Values are relative to 100% baseline (B).

Figure 6: Cytokine secretion of (a) TNF- α , (b) IL-8, and (c) IL-6 from primary HBECs after exposure to filtered air (FA), neat diesel emissions (nDE), residual diesel emissions (rDE) for 30 and 60 minutes and cigarette smoke condensate (CSC) for 3 hours. * indicates a statistically significant difference ($P < 0.05$). Error bars indicate SEM ($n = 3$). Concentrations are corrected for cell viability.

Figure 7: Gene expression levels of CYP1A1 (a), HO-1 (b), TNF α (c) and IL-8 (d) from primary HBECs after exposure to filtered air (FA), neat diesel emissions (nDE), residual diesel emissions (rDE) for 30 and 60 minutes, and cigarette smoke condensate (CSC) for 3 hours. Gene expression is relative to the baseline control group and relative expression is normalized to GAPDH. * indicates a statistically significant difference ($P < 0.05$). Error bars indicate SEM ($n = 3$).

Table 1: Average particle concentration, mass concentration and gas concentration (NO, NO₂, CO and CO₂) during 30 and 60 minute exposures of primary HBECs to nDE and rDE at ALI.